A multibiomarker approach in juvenile turbot, Scophthalmus maximus, exposed to contaminated sediments

Elodie Kerambrun, Françoise Henry, A. Marechal, W. Sanchez, I. Filipuci, C. Minier, Rachid Amara

A multibiomarker approach in juvenile turbot, *Scophthalmus maximus*, exposed to contaminated sediments

Kerambrun, E.[1,2,3,*], Henry, F. [1,2,3], Marechal, A.[4], Sanchez, W.[5], Filipuci, I. [1,2,3], Minier, C.[4], Amara, R.[1,2,3]

[1] Univ Lille Nord de France
[2] ULCO, LOG, F-62930 Wimereux, France
[3] CNRS, UMR 8187, F-62930 Wimereux, France
[4] Laboratory of Ecotoxicology, Université du Havre, F- 76058, Le Havre, France
[5] Unité d'écotoxicologie *in vitro* et *in vivo*, Institut National de l'Environnement Industriel et des Risques (INERIS), BP 2, F-60550 Verneuil en Halatte, France

* Corresponding author: Elodie Kerambrun

elodie.kerambrun@univ-littoral.fr

Abstract

Juvenile turbots were exposed in laboratory condition to a mixture of chemical contaminants associated with harbour and estuarine sediments during 7 and 21 days. Several molecular biomarkers of exposure were then measured in fish liver: two biotransformation parameters [ethoxyresorufin-O-deethylase (EROD) and phase II glutathione S-transferase (GST) activities] and an antioxidant enzyme activity [catalase (CAT)]. Modifications at the histological level were analysed by the measurement of the numbers and size of melanomacrophage centers (MMCs) and disturbances in the immune function by the measurement of cytokine transforming growth factor-beta (TGF-β) and development of the thymus. The responses of theses molecular and immunological biomarkers were put in relation with metal and PAH concentrations measured in sediments and with turbot physiological performance analyzed in a previous study on the same fish (growth rates, condition factor K, RNA:DNA ratio and lipid index). Whereas no difference was found in thymus analysis, some molecular and immunological responses were observed in fish exposed to contaminated sediments. Weak relationships between molecular biomarkers responses and PAHs concentrations were recorded whereas their responses were significantly correlated with some metals. MMC and aggregates were weakly related with chemical contaminant whereas some significant correlations were found between TGF-b1 responses and some metal concentrations. However, molecular and immunological biomarkers were weakly related with fish physiological damages since low responses were observed in the condition which led to the lowest growth and condition indices. These data suggest the complexicty of cause-effect relationships between exposure to pollutants, metabolisms and health damages. Precautions should be considered in the use of molecular and immunological biomarkers alone in biomonitoring programs. Their complementary used with physiological biomarkers as fish growth and condition indices could improve their utilization.

Keywords : EROD, CAT, TGF-b, melanomacrophage centers, fish health, metals, PAHs

1. Introduction

In most of contaminated environments, such harbour areas, organisms are exposed to mixtures of pollutants, whose synergistic or antagonist effects are hardly interpreted and predicted exclusively from the chemical analyses (Regoli et al., 2004). That's why, in recent years different biological tests have been developed representing low-cost tools to evaluate biological responses to environmental pollution at the molecular, cellular and organismic level (McCarthy et al., 1990; Shugart et al., 1992; Viarengo et al., 1996). However, it is still complex and difficult to predict the biological effects caused by different classes of chemicals during co-exposures when reciprocal interactions, cascade and indirect mechanisms can both enhance or suppress the expected responses (Benedetti et al., 2007).

In a previous study, we have analysed the responses of several molecular and physiological biomarkers of juvenile sea bass and turbot caged in a polluted harbour (Kerambrun et al., 2011a,b). Results of this study suggest the influence of biotic and/or abiotic factors on juvenile turbot responses to the chemical contamination. Indeed, release and bioavailability of contaminants are highly regulated by hydrodynamics, biogeochemical processes and environmental conditions of the system (Eggleton and Thomas, 2004). Moreover, many studies reported the influence of various biotic and environmental factors on biomarker responses measured in aquatic organisms (Sanchez et al., 2008). Laboratory approaches in ecotoxicology could represent an advantage for establishing clearer cause and effect linkages between contaminants and toxicity, as well as the mechanistic basis for toxicity. Therefore, the use of sediment toxicity bioassay complementarily with caging study could lead to a better understanding of the confounding factors in the assessment of the effects of chemical contaminants.

Since single biomarkers cannot reflect the impairment of organism health conditions, the use of different biomarkers than can both signal exposure to contaminants and quantify their effects on the health of organisms, enables a more comprehensive and integrative assessment of environmental quality (Fonseca et al., 2011). In the present study, a multi-biomarker approach was used to analyze the effects of contaminants associated to sediments on juvenile turbot in laboratory conditions. Turbot species was chosen because it is a commercial fish with a wide range of distribution along the European coast. Moreover, this species maintains a close association with sediments for food and protection and is therefore more likely to be exposed to sediment-associated pollutants (Besselink et al., 1998; Kilemade et al., 2009). Sediment was sampled in a northern French harbour, Boulogne sur Mer, which is an intensively developed and industrialised harbour impacted by municipal and industrial discharges, fishing and shipping activities, and marinas. We

have also chosen to sample sediment in an anthropogenic estuary, the Seine. This estuary remains a highly productive ecosystem, which provides a nursery for numerous marine fish in spite of heavy organic and metallic contamination and human activities (Dauvin and Desroy, 2007).

Among the numerous biological responses, those based on the molecular and cellular level can represent the earliest warning signals of environmental disturbance (Depledge, 1994). Several molecular biomarkers of exposure were therefore chosen since they are frequently used in ecotoxicological studies: biotransformation enzymes [phase I ethoxyreesorufin-O-deethylase (EROD) and phase II glutathione S-transferase (GST) activities] that metabolize xenobiotics and an antioxidant enzyme activities [catalase (CAT)] which reduce cellular damage resulting from reactive oxygen species (ROS). As a number of environmental chemicals have the potential to impair components of the immune system (Wester et al., 1994), disturbances in the immune function were analysed by the measurement of the numbers and size of melanomacrophage centers (MMCs) and the cytokine transforming growth factor-beta (TGF-β) and development of the thymus. The responses of theses molecular and immunological biomarkers were put in relation with turbot growth and condition measured in a previous study (Kerambrun et al, 2011c). We used three condition indices: the Fulton's K condition factor; the RNA:DNA ratio which is used in numerous studies as indices for nutritional condition and growth assessment in larvae and juvenile fish (Buckley, 1984; Gwack and Tanaka, 2001; Amara et al., 2009); and a lipid storage index based on the ratio of the quantity of triacylglycerols (TAG; reserve lipids) to the quantity of sterols (ST; structural lipids) in fish (Amara et al., 2007).

The main objective of this study was to assess and compare the responses of these biomarkers measured on turbot exposed to a mixture of chemical contaminant associated with harbour and estuarine sediments. This multibiomarker approach intended to explore a wide range of fish responses to pollution from the sub-cellular level to the general condition of fish. These laboratory data were compared with those obtained from our previous caging study to analyse the predictability of these biomarkers in field situation.

2. Material and Methods

This experiment was conducted in accordance with European Commission recommendation 2007/526/EC, on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes.

2.1. Sediment exposure conditions

Sediments were collected from different sites located along the French coast of the Eastern English Channel at the same time in February 2010. These sediments were sampled from three stations in a harbour in northern France (Boulogne-sur-Mer: BSM); from an anthropogenic French estuary (the Seine) and from a reference site (Fig. 1). The sediment was collected using a Van Veen grab (250 cm^2 sampling area) in three different locations in the harbour: station A in the front, and stations B and C in the inner part. Estuarine inter-tidal sediment was collected at low tide from the north bank of the Seine estuary. The last sample was taken at low tide from Wimereux beach, used as the reference site.

Four month old turbots, *Scophthalmus maximus,* (weight : 6.83 ± 0.72 g) were obtained from a hatchery (France Turbot) and acclimatised in two clean tanks (160 L) in semi-static conditions for two weeks. Before the beginning of the experiment, each fish was anaesthetised in a 200 $\mu g.L^{-1}$ 2−phenoxyethanol solution, weighed (0.01 g accuracy), measured for total length (0.1 mm accuracy) and individually marked (Visual Implant Tag, 1.2 mm x 2.7 mm, Northwest Marine Technology). The experimental 21-day assay consisted of a static water system of 37 L capacity glass tanks, in which 5 L of sediment and 25 L of clean seawater were allocated. The assay was performed in duplicate for the five different sediments, thus ten tanks were used. The daily feeding amount was maintained at approximately 1% of the total fish weight. No mortality was observed in any of the exposure tanks. Temperature (13.8 ± 0.1 °C), salinity (32.1 ± 0.2 PSU), pH (7.90 ± 0.03) and oxygen levels (8.01 ± 0.10 $mg.L^{-1}$) were constant and similar in the different exposure tanks throughout the experimental assay.

Fifteen fish (7-8 per duplicate tank) per treatment group was sampled after 7 and 21 days of exposure and anaesthetised with 2-phenoxyethanol. The turbots were identified (tagged), weighed and measured. From each fish, 1 mL of peripheral blood was withdrawn from the caudal vein with a lithium heparinised vacutainer, centrifuged 7 min at 700g, 4°C. Supernatant was transferred into microtubes and conserved at 80°C until further analyses. The spleen and a large piece of the head including the thymus were collected and conserved in 4% buffered formaldehyde while the liver was frozen in liquid nitrogen and preserved at -80°C. Muscles fragments were stored at -20°C and otoliths (sagittae) were extracted and preserved in ethanol (95%).

2.3. Sediment analysis

In order to determine selected metals (Cd, Cr, Cu, Mn, Ni, Pb, V and Zn) in the total fractions, the sediments were dried in an oven at 40 °C to constant weight and then ground into powder. For

the determination of total metals, about 0.250 g of ground sediment were digested with HF (Suprapur, Merck) at 110 °C for 48 h followed by a mixture of concentrated acids HCl:HNO_3 (3:1, v:v, Suprapur Merck) at 120 °C for 24 h. This operation was repeated once. For quality assurance, reagents blanks, sample replicates and standard reference materials (MESS−3 and PACS−2, National Research Council Canada) were used to assess the accuracy and precision of the analyses. In all cases, the recovery efficiency was better than 85% for the total digestion of standard reference materials.

The persistent organic pollutants, including PAHs (EPA's 16 priority PAHs) and PCBs (7 congeners) were analysed. Briefly, organic compounds were extracted from 2 g of dried sediment by a microwave oven (120 °C for 15 min, 1200 W), assisted with a 40 mL mixture of acetone and hexane (1:1, v:v). The solvent was evaporated under a stream of nitrogen in a TurboVap, and then concentrated to 1 mL of hexane. Simultaneous determination of PAHs and PCBs was performed on a gas chromatography−mass spectrometer (GC−MS, VARIAN, CP 3800 – 1200 MS TQ). A ZB−MultiResidue column (30 m, 0.25 mm, 0.25 µm) was used (Phenomenex). Identification of PAH compounds and PCB congeners was based on the comparison of their GC−retention times and their mass spectrum, with appropriate individual standards. Total Hg was measured in dry and ground sediment samples (without any pre−treatment) by means of atomic absorption spectroscopy (AAS) using an AMA 254 solid phase Hg−Analyzer (Altec Ltd., Prague, Czech Republic) (Ouddane et al., 2008). Mean recovery for total Hg was between 80 and 100% for certified estuarine sediment IAEA−405 (IAEA, Vienna, Austria).

2.4. Biological analysis

2.4.1. Molecular biomarkers analysis

Livers were homogenised in an ice-cold phosphate buffer (0.1 M, pH 7.8) with 20% glycerol and 0.2 mM phenylmethylsulfonyl fluoride as a serine protease inhibitor. The homogenates were centrifuged at 10,000 g at 4 °C, for 15 min and the post-mitochondrial fractions were used for biochemical assays. Total protein concentrations were determined using the method of Bradford (1976) with bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard.

Ethoxyresorufin-O-deethylase activity (EROD) was determined following the hydroxylation of 7-ethoxyresorufin by the method of Flammarion et al. (1998). The reaction mixture consisted of a phosphate buffer (0.1 M, pH 6.5), 7-ethoxyresorufin (8 µM) and NADPH (0.5 mM). The change in fluorescence was recorded (excitation wavelength 530 nm, emission wavelength 585 nm) and enzyme activity calculated as $pmol.min^{-1}.mg^{-1}$ protein using a Resorufin standard.

Glutathione S-transferase activity (GST) was determined following the conjugation of reduced glutathione with CDNB by the method of Habig et al. (1974). The reaction mixture consisted of a phosphate buffer (0.1 M, pH 6.5), reduced glutathione (1 mM) and CDNB (1 mM). The change in absorbance was recorded at 340 nm and enzyme activity calculated as $\mu mol.min^{-1}.mg^{-1}$ protein using GST standard.

Catalase activity (CAT) was determined by the method of Babo and Vasseur (1992). In brief, the assay mixture consisted of a phosphate buffer (100 mM pH 6.5) and H_2O_2 (28 mM). Change in absorbance was recorded at 240 nm. CAT activity was calculated in terms of $\mu mol.min^{-1}.mg^{-1}$ protein, using bovine erythrocyte Catalase as standard.

2.4.2. Immunological biomarkers

Thymus and spleen were processed for histological analysis. Prior to sectioning, fish heads were decalcified in 10% formic acid, 10% dinatrium citrate aqueous solution (Sigma aldrich) for 7 days. Samples were embedded in paraffin, and entirely cut into 5 µm sections. Slides were colored with hematoxylin eosin safran and observed under light microscope. Images of the tissues sections were finally taken and analyzed by a computerized image analysis system (Qwin, Leica). Thymus volume was deduced from the addition of thymic areas multiplied by the total thickness of sections. The cortex-medulla ratio was calculated from the areas of these two thymic regions. Five sections from the median part of the spleen were randomly selected and analyzed. Relative abundance of melanomacrophage centers (MMC) and dark-brown aggregates were quantified by dividing the numbers of the corresponding foci by the area of the section.

Plasmatic levels of activated TGF-b1 were determinated immunologically (Invitrogen, KAC1688, Camarillo, Spain) according to manufacturer instructions.

2.4.3. Physiological biomarkers

The somatic growth in length and in weigth, the otolith recent growth, the Fulton's K condition index, the RNA:DNA ratio and the lipid index, based on the TAG:ST ratio, were analysed in fish following the 21 days exposure as described in Kerambrun et al., 2011.

2.5. Statistical analysis

Mean comparisons of molecular biomarker responses between the five conditions of exposure were analysed using one-way ANOVA, followed by post-hoc Tukey tests. For the density of splenic MMCs and aggregates, and TGF-b1 levels, statistical significance was analyzed by

Kruskal-Wallis rank sum test which was followed by Kruskal multiple comparison test. Differences were considered significant when $P < 0.05$.

Using the data from all individual collected and chemical contamination analysed in sediment, Pearson correlation between biomarkers and chemical contaminant were computed to examine the statistic links between parameters. Significance of the correlations was determined for each variable.

As molecular, immunological and physiological biomarkers were analysed at t_7 and t_{21} on the same fish, a Pearson product moment correlation matrix was computed using data from all the individuals exposed, to examine the statistic links between parameters.

Coefficients of variation (CV) for molecular, immunological and physiological biomarkers were calculated to analyse the heterogeneity of each variable using the formula :

$CV_i = 100 \times SD_i / M_i$.

Where SD_i and M_i represented the variable standard deviation and mean, respectively, for the condition i. Final CV of each biological parameter were obtained by the mean of the five CV_i (one per condition).

3. **Results**

3.1. Chemical contamination in sediment

No measurable PCB congeners were detected above 0.01 $mg.kg^{-1}$ in any of the sediments tested. The metal concentrations measured in the total fraction of sediments are reported in Fig. 2. As expected, the reference sediment was the least enriched by metal compounds. The A sediment presented concentration in metals about 3 time higher than in reference sediment but showed lower metal concentrations compared to the two other harbour sediments. The B and C sediments showed different levels of contamination among metals. The B sediment showed the highest levels of Cd, Mn, and Pb, which were approximately 16, 33 and 12 times higher than reference sediment, respectively. In contrast, Cu and Zn were more abundant in the C sediment, with concentrations respectively 140 and 43 times higher than those measured in the reference site. All metal concentrations were higher in the Seine sediment compared to the A sediment, but they remained lower than the two other harbour sediments, except for the chromium concentration, which was of the same order of magnitude.

No PAH was detected in reference and A sediments. Higher total PAH concentrations were found in the C sediment (2.44 ± 0.95 $mg.kg^{-1}$) compared to the B one (1.27 ± 0.50 $mg.kg^{-1}$). The

total PAH concentration measured in the Seine sediment (1.63 ± 0.64 mg.kg^{-1}) was between those measured in the B and the C sediments. Among the different aromatic compounds detected in this sediment, PAHs with intermediate-high molecular weight were dominant.

3.2. Molecular biomarker responses

Molecular biomarker responses analysed following the 7 and 21 days exposure are presented in Fig. 3. A significant increase of EROD activities was observed at t_7 in fish exposed to A, B and Seine sediments compared to t_0. These EROD activities measured at t_7 were also significantly higher in turbot exposed to A sediment compared to reference sediment and exposed to B sediment compared to the four other conditions. Following 21 days exposure, EROD activities were only higher in fish exposed to C sediment compared to t_0 and in fish exposed to Seine sediment compared to t_0 and reference sediment. No significant difference was observed for GST activities at t_7 and a significant increase was found at t_{21} in turbot exposed to B sediment compared to other conditions. On the opposite, significant lower GST activities were observed in fish exposed to C sediment compared to reference sediment. A significant increase of CAT activities was observed in fish exposed to the four contaminated sediments during 7 days compared to t_0 and reference sediment. Indeed, at t_7, CAT activities increase significantly from C and Seine condition to A condition and then to B condition. Following 21 days exposure, the only significant increase in CAT activities was observed in fish exposed to B sediment.

3.3. Imunnological biomarker responses

TGF-b1 plasma concentration was constant in control fish throughout the 21-d experiment with values of 34-36 ng.mL^{-1} (Fig. 4). Similar values were obtained in fish exposed to contaminated sediments for 7 days although a low but significant decrease could be measured for fish exposed to the Seine and the A sediments. However, after 21 days of exposure, all sediment-exposed fish showed a slight and non-significant increase in TGF-b1 plasma concentration when compared value obtained after 7 days of exposure. This rise led to concentrations that were higher than 40 ng/mL in condition B, C and with the Seine estuary sediments.

Relative abundance of spleen melanomacrophage centers (MMCs) was low in the control fish (2 MMCs.mm^{-2}, Fig. 5). Interestingly, starvation for 7 or 21 days did not result in any change in MMC numbers (not shown). However, with the exception of condition B, MMC density was 3 to 4 times more elevated in fish exposed for 21 days to harbour (condition A and C) and Seine Estuary

sediments. A similar effect could be assessed when measuring the relative abundance of brown aggregates within the spleen tissues. Although no statistically significant differences could be measured for the latter, aggregate density was 2-3 more elevated in tissues from fish exposed to contaminated sediments (Fig. 5).

The thymus volume of fish exposed to harbour sediment (condition C) was not significantly different from the control group after 21 days of exposure (622 μm^2 ± 366 versus 722 μm^2 ± 190). Similarly, no significant difference in thymic cortex medulla ratio could be measured between these two conditions (1.40 ± 0.28 versus 1.39 ± 0.2). Thus, no variation in T cells maturation process could be assessed during the experiment.

3.4. Correlations of molecular, immunological and physiological biomarkers with chemical contaminants.

In order to compare the responses of molecular, immunological and physiological biomarkers to the different level of chemical contaminants found in sediments, correlations between biological parameters, the eight metal compounds analysed and the total PAH concentrations were performed. The coefficients of correlation are presented in table 1. At t_7, EROD and CAT were significantly correlated with each other and with Cd, Hg, Cr, Mn, Ni, Pb and V whereas GST presented no significant correlation with chemical contaminants. Following 21 days exposure, EROD presented significant correlations with Cr, Hg, Ni, Pb, V and total PAHs, GST with Cd, Cu and Mn, and CAT with Cd, Mn, Ni, Pb and V. With the exception of the correlation found between GST and Cu, all significant correlations found with these molecular biomarkers were positively related with chemical contaminants. No correlation could be found between the occurrence of spleen MMC and any measured contaminant while TGF-b1 plasma concentration appeared significantly related to some of the metals such as Cd, Cr, Hg, Mn, Ni and Pb.

3.5. Relationships between molecular, immunological and physiological biomarker responses.

Correlations between molecular, immunological and physiological biomarkers were also performed to test the existence of significant links between these different parameters, and to evaluate the strength of these relationships. Results are presented in table 2. At t_7, only CAT activities presented significant correlations with EROD, GW, GL and K but R values were lower than 0.30. At t_{21}, EROD and CAT activities were found be significantly and positively correlated.

Negative significant correlations were observed between EROD activities and the RNA:DNA ratios. CAT activities were found significantly correlated with GW, GL and K. For the measured immunological parameters, only TGF-b1 plasma concentration showed a correlation with the K index.

3.6. Variability of measurements

Coefficients of variation (CV) for molecular and physiological biomarkers were calculated to analyse the heterogeneity of each variable. As growth rates ranged with negative and positive values, CV could not been estimated for these variables. The lowest coefficients of variation were observed for the K index (CV_K=5.32%) the otolith recent growth (CV_{RG} = 9.10%) and TGF-b1 ($CV_{TGF\text{-}b1}$ = 9.31%). The CV was quite similar between the three molecular biomarkers (CV_{EROD} = 18.4%, CV_{GST}=18.1% and CV_{CAT} = 21.7%) and highest values were found for RNA:DNA ratios ($CV_{RNA:DNA}$ = 32.0%), MMCs (CV_{MMC} = 56.6%), aggregates (CV_{agg} = 56.7%) and for TAG:ST ratios ($CV_{TAG:ST}$ = 91.1%).

4. Discussion

4.1. Molecular biomarker responses

Differences in EROD, GST and CAT activities were observed in turbot exposed to the four contaminated sediments compared to reference. Highest responses were observed in fish exposed to B sediment while sediment C presented highest concentrations of most of chemical contaminants. These molecular biomarkers are frequently used in ecotoxicological studies due to their sensitivity to different classes of pollutants (Van der Oost et al., 2003). For example, Kilemade et al. (2009) have analysed the EROD responses in hatchery-reared 3 months turbot exposed to sediments from two contaminated sites within Cork harbour which presented contaminants similar to ours. These authors found a significant increase of EROD activities after 7, 14 and 21 days of exposure to these contaminated sediments compared to their reference. In the same way, higher GST activities were observed in flounder exposed to sediment from the harbour of Venice (Vigano et al., 2001) and an increase of GST and CAT was found in neotropical fish *Prochilodus lineatus* exposed to sediment from an urban area (Almeida et al., 2005). However, as sediments are composed by a mixture of chemical contaminants, it is still difficult to know what pollutant is responsible for theses molecular biomarkers responses. In this present study, we have analysed their correlations with the different chemical contaminants found in sediments. Even metal and PAH are only some chemical compounds which are present in such contaminated sediment, they represented two important groups of contaminant whose chemical constitution makes them prone to adsorption by particles (Magnusson et al., 1996). Co-contamination with complex mixtures of metals and polycyclic aromatic hydrocarbons (PAH) is a common environmental problem with multiple biological consequences, particularly to the enzyme systems and metabolism in the body (Korashy and El-Kadi, 2004)

Among the three molecular biomarkers analysed, GST presented the least significant correlations with contaminants with only significant positive correlations with Cd and Mn at t_{21} and negative correlation with Cu. More significant correlations were found for EROD and CAT activities. In particular, at t_7, EROD and CAT activities showed relatively strong positive relationships with Cd, Mn and Pb. However, weak correlations were found in EROD and CAT activities with Cu, Zn in spite of their relatively high concentrations in C sediment. Whereas many studies have observed an induction of detoxification parameters or antioxidant enzymes in fish under organic contamination as PAHs (Achuba and Osakwe, 2003; Simonato et al., 2008; Nahrgang et al. 2010), these three molecular biomarkers were weakly related with PAH

concentrations found in sediments. The only significant correlation was observed for EROD at t_{21}. In particular, few responses were observed in fish exposed to sediment C whereas it displayed the highest concentration of PAHs. Antagonist effects of pollutants could represent an explanation of these low responses of molecular biomarkers. In particular different studies have observed metal inhibitions of these enzyme activities (Pandey, 2008; Atli and Canli, 2010; Cao et al., 2010). For example, the induction of EROD and CAT activity in Antarctic fish *Trematomus bernacchii* exposed to benzo(a)pyrene, was greatly suppressed by Cu (Benedetti et al., 2007). In the same way, liver microsomal EROD activity was significantly inhibited in sea bass after *in vitro* exposure to Cu and Zn (Oliveira et al., 2004). Metals can alter the activity of enzymes by binding to their functional groups but also by altering protein turnover (Viarengo, 1996; Oliveira et al., 2004). Moreover, metal do not act on the same way in organisms since essential metal tend to be highly regulated compared to non essential metals (Fernandes et al., 2007). Under these circumstances the effect of mixtures is more difficult to predict. Moreover, our conclusions from this correlation study have to be moderated since other contaminants, not measured in this study, could have influenced the biomarkers responses. The responses observed could be the results of the sum-up of this pollutant mixture. Other metal scavengers, i.e. metallothioneins, are also known to perform valid metal detoxification (Viarengo & Nott, 1993 ; Schröder et al., 2000 ; Triebskorn et al., 2002). However, in this present study we were limited by the liver size of such juvenile fish. Moreover, metallothionein responses to chemical contaminants were found to present a quite high variability especially by the effects of biotic and abiotic factors on their synthesis (Amiard et al., 2006).

Differences in molecular biomarkers responses were also observed between the two times of exposure. Differences in GST activities were only observed at t_{21} whereas EROD and CAT responses were lower at t_{21} compared to t_7. Differences in EROD activities among the duration of time was also observed in turbot exposed to oil produced water (Stephens et al., 2000) and in the antioxidant system of freshwater fish *Oreochromis niloticus* exposed to different metals (Atli and Canli, 2005). In their study, Wu et al. (2005) have examined the time-integrated response of various biomarkers. They propose a typical profile which included a latent period, an induction period related to the concentration of contaminants and a decline of the response refereed as adaptation period if the exposure is prolonged. The time required for the occurrence of each process may be species-specific, contaminant-specific and dependent upon the level of biological response. The results of the present study suggest that GST would have a slow induction period while EROD and CAT would have a fast recovery period for this type of sediment contamination. In the present study, molecular biomarkers responses were analysed at t_7 and t_{21}, however if we have considered other times of exposure, different molecular responses could have been observed.

These time-dependent responses could be problematic in field situation resulting by an underestimation of the level of contaminants in the field (Wu et al., 2005).

4.2. Relationships between molecular and physiological biomarkers.

The strategy using molecular responses in environmental assessments relies upon the fact that interactions between pollutants and molecular functions may indicate or lead to vital disturbances in for example reproduction success, growth or survival (Magnusson et al., 1996). The assumption for this fact is that there are metabolic costs associated with the synthesis of these proteins or with detoxification processes (Rose et al., 2006). In the present study, we used physiological biomarker results from a previous study (Kerambrun et al., 2011c) to know if variations in EROD, GST or CAT activities could be related with variations in turbot growth or condition.

Whereas EROD and CAT showed the highest responses at t_7, low relationship was found between molecular biomarkers responses at t_7 and the fish physiological performance. At t_{21}, EROD increase was found significantly and negatively related with RNA:DNA ratios and CAT with both specific growth rates and the Fulton's K index. However, this relationship remained relatively low with correlation coefficient about 30%. The main difference between molecular and physiological biomarkers was in the responses observed in turbot exposed to C sediment. Indeed, few differences of molecular biomarkers were observed in fish exposed to HC sediment while high difference was observed for the condition B. On the opposite the lowest responses of physiological biomarkers were observed for the condition C. As said previously, B and C sediment presented different level of contamination among the metal. This fact supports the hypothesis suggests previously of potential antagonist effects of the different chemical contaminant present in sediments on molecular biomarkers activities. The analyse of relationships between molecular and physiological biomarker responses suggest that based only on biomarkers responses, biological effects of exposure to C sediment would be underestimated.

The biomarker responses observed in this laboratory study could be compared with those obtained in a previous study in which same age turbot were caged during 38 days in 2009 in the same harbour, at station A, B and C (Kerambrun et al., 2011a,b). In these previous studies, significant decreases of growth, Fulton's index and TAG:ST ratio were found in turbot caged in station B compared to station A which corroborate results from this present study. Some differences were observed for molecular biomarker responses. While a significant increase of EROD activities was observed in fish caged in station B, no significant difference was observed in

turbot exposed to sediment B for 21 days whereas metal and PAH concentrations were similar in both studies. CAT activities were lower in fish caged in station B whereas a significant increase was observed in fish exposed to sediment B compared to A. Low biomarker responses were observed for condition C in the present study while fish were found dead in caging station C. However, chemical concentrations observed in the caging study was about 2 times higher in station C than in the present study for Mn, Pb, Zn and total PAHs. Several additional factors could be suggested to explain differences between the two kind of studies as the caging physiological stress (Oikari et al., 2007), the additional seawater contamination in the water column or the time of exposure.

4.3. Immunological biomarker responses

No effect could be demonstrated in terms of thymic involution or of default in T cells maturation in juvenile turbot during the 21 days of exposure in this study. Similar results were reported for flounder exposed to 500 µg of TCDD .kg^{-1} bw (Grinwis et al., 2000). This could be attributed to the relative short duration of the experiment or to a relative resistance of the fish. As cells maturation is a relatively slow process, alterations at the histological level may only be prevalent after a long time of exposure. Furthermore, the decrease of thymus size appears to be a species-specific marker. Wester and collaborators showed that upon exposure to TBTO, only guppy (*Poecilia reticulata*) showed a thymic involution, whereas medaka (*Oryzias latipes*) did not (Wester and Canton, 1987; Wester *et al*., 1990).

In this study, the density of MMCs in the spleen of the fish did not show much variation. However, values were elevated after 21 days of exposure to contaminated sediments. It is assumed that they play an important role in the phagocytosis of cellular debris (Wolke, 1992; Leknes, 2007). MMCs can also be indicative of stimulation of the innate immune system due to the fact that macrophages are important actors of the nonspecific defense notably during inflammation. Nevertheless, its significance is unclear (Couillard et al., 1999) and MMC density was not significantly correlated to any physiological traits including growth and condition index of the fish used in the study. Its ability to be predictive of adverse effects thus deserves further investigation, especially during fish development. The increase number of brown aggregates paralleled the high MMCs density in spleen of fish exposed to sediments. These aggregates might be breakdown products which remained in tissues after the disappearance of macrophages and maybe indicative of lipid peroxidation (Wolke, 1992). Both MMCs and brown aggregates might be closely related.

Effect on the immune system was further indicated by the low increase in plasma TGF-b1 in all fish except control fish after 21 days of exposure to contaminated sediments. According to Harms et al. (2000), an increase of TGF-b1 level suggests an immunosuppressive effect due to its fundamental ability to turn off several components of immunity. No such conclusion could be derived from the present work. However, the modulation TGF-b1 plasma concentration is similar to the changes in MMCs and, together, may indicate some changes that might be important for the developing fish.

Immunological measurements were not related to effects on growth or condition indices. It could be hypothesized that immunological effects are specific for a system and cells whereas the general condition reflects either the energy supply or its allocation to diverse systems. In this respect immunological development of the organisms could be a priority for the organisms during development thus not directly correlated to general condition. No obvious relationships were noted between the immunological parameters and the measured contaminants. Only the TGF-b1 plasma concentration was correlated to some of the metals. Recent studies report that a wide range of pollutants can induce greater secretion of this cytokine (Sonne et al., 2007) and the observed correlation does not infer a direct relationship. On the contrary, some unmeasured contaminants may have contributed to the slight responses of the immunological system.

Although the measured effects were low, studies on the immune or neuro-endocrine system may be important. Not only they represent an important system necessary for the good functioning of the organisms, but alteration may also lead to profound and lasting effects resulting increased susceptibility, diseases, or death in the long term.

4.4. Conclusion

These data confirm complex cause-effect relationships between exposure to pollutants, metabolisms and heath damages. Molecular biomarkers were weakly related to fish physiological damage and their responses were not similar between this laboratory experiment and our previous caging study. The inconsistency of molecular responses measured in this work suggests that variations of molecular biomarkers can be hardly predictable in field situation. Indeed, field organisms are exposed to pollutant mixtures including metals and PAHs, which could cause synergistic or antagonist effects on molecular biomarkers responses whereas immunological and physiological biomarkers appears to be more predictive of the adverse effects of chemical contaminant. However, immunological biomarkers appear to be weakly related with chemical contaminants found in sediments and fish physiological performance even TGF-b1 showed some

significant correlations. On the other hand, the inherent natural variability of physiological biomarkers and their sensitivity to several biotic or abiotic factors (sediment grain size, currents, food availability…) occurring in field situation could lead to a misunderstanding of biological effects of chemical contaminants. This study suggests that molecular and immunological biomarkers should not be used alone in biomonitoring programs since interactions between chemical contaminants could alter their responses and lead to an underestimation of biological effects. Their complementary use with growth and condition indices could lead to a better understanding of the adverse effects of chemical contaminants on fish health.

Acknowledgements

This work was supported by post Grenelle programme 190, DEVIL of the French ministry for ecology and the Franco−British INTERREG IVA European project, DIESE. We would like to thank Prescilla PERRICHON, Lucie COURCOT, Vincent CORNILLE and Michel LAREAL for their help during the experiment assay.

Figure legends.

Fig. 1. Locations of the three sediment sampling sites (Reference, the Seine estuary and harbour of Boulogne sur Mer) and the three stations in the harbour (A, B and C).

Fig. 2. Metal concentrations ($mg.kg^{-1}$ dry weight) in the five sediments (Ref, Seine, A, B and C)

Fig. 3. Differences in Ethoxyresorufin-O-deethylase (EROD), Glutathione S-transferase (GST) and Catalase (CAT) activities of turbot exposed to the five sediments (Ref, Seine, A, B and C) during 7 (□) and 21 (■) days. n = 15.

(^{0}), (^{1}), (^{2}), (^{3}), (^{4}), (^{5}) represent significant difference ($p < 0.05$) compared to "t_0", REF, Seine, A, B, C.

Fig. 4. Differences in plasmatic levels of TGF-b1 ($ng.ml^{-1}$) of turbot exposed to the five sediments (Ref, Seine, A, B and C) during 7 (□) and 21 (■) days. n= 3.

(^{1}) represent significant difference ($p < 0.05$) compared to REF.

Fig. 5. Differences in relative abundance of melanomacrophage centers (MMC) and dark-brown aggregates of turbot exposed to the five sediments (Ref, Seine, A, B and C) during 21 days. n=8.

(^{1}) represent significant difference ($p < 0.05$) compared to REF.

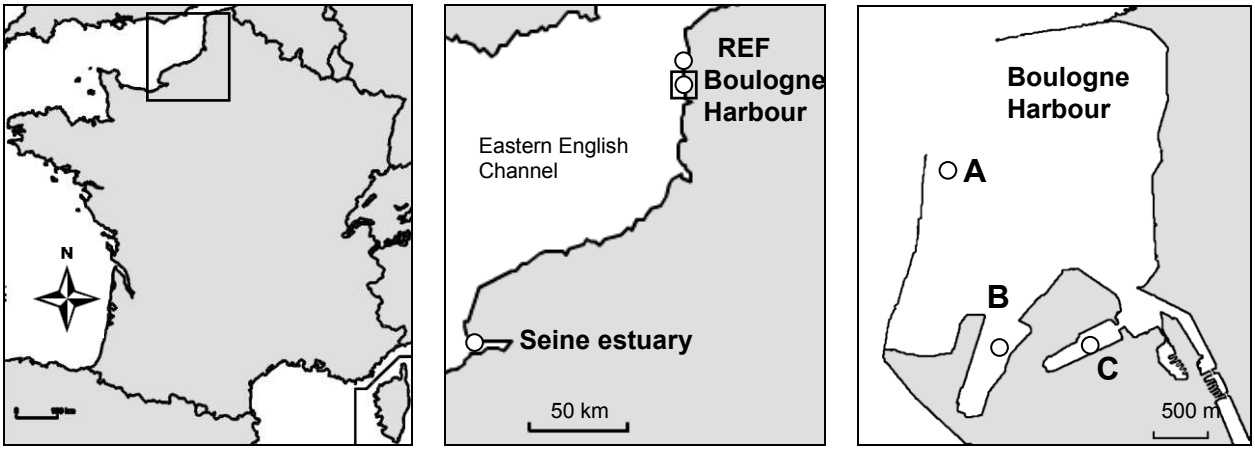
N
REF
Boulogne
Harbour
Eastern English
Channel
Seine estuary
50 km
Boulogne
Harbour
A
B
C
500 m

Fig. 1

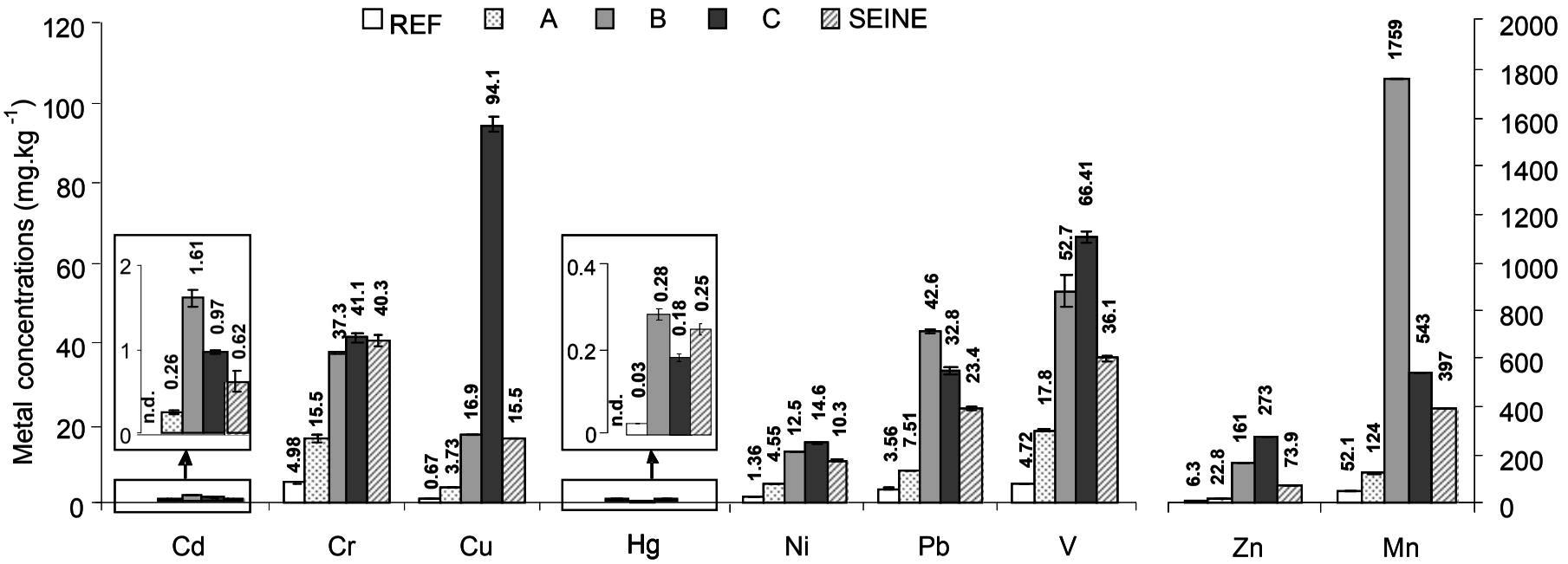
REF
A
B
C
SEINE
Metal concentrations (mg.kg-1)
Cd
Cr
Cu
Hg
Ni
Pb
V
Zn
Mn
n.d.
0.26
1.61
0.97
0.62
4.98
15.5
37.3
41.1
40.3
0.67
3.73
16.9
94.1
15.5
n.d.
0.03
0.28
0.18
0.25
1.36
4.55
12.5
14.6
10.3
3.56
7.51
42.6
32.8
23.4
4.72
17.8
52.7
66.41
36.1
6.3
22.8
161
273
73.9
52.1
124
1759
543
397

Fig.2

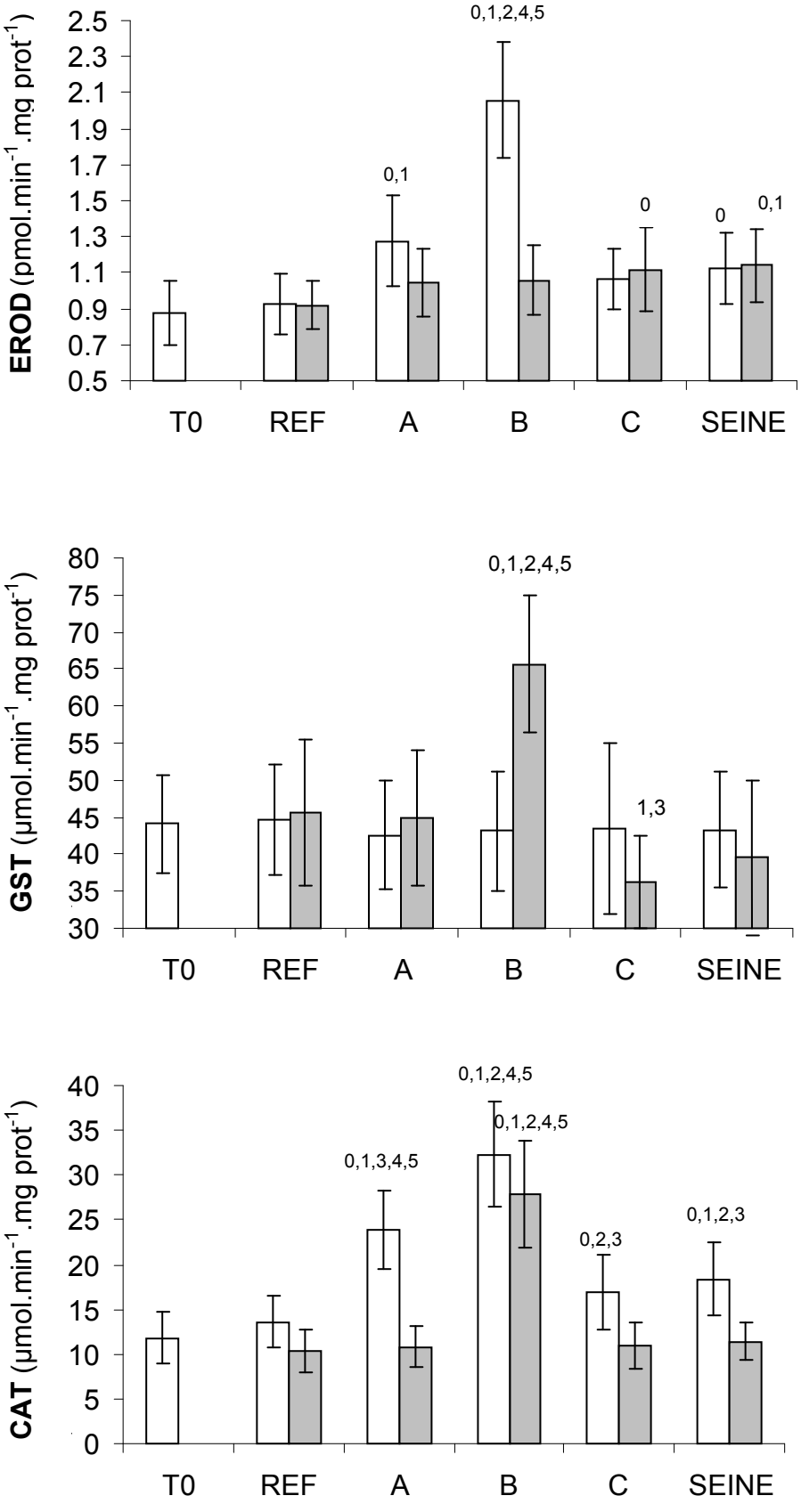
EROD (pmol.min-1.mg prot-1)
2.5
2.3
2.1
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0,1,2,4,5
0,1
0
0
0,1
T0
REF
A
B
C
SEINE
GST (µmol.min-1.mg prot-1)
80
75
70
65
60
55
50
45
40
35
30
0,1,2,4,5
1,3
T0
REF
A
B
C
SEINE
CAT (µmol.min-1.mg prot-1)
40
35
30
25
20
15
10
5
0
0,1,2,4,5
0,1,2,4,5
0,1,3,4,5
0,2,3
0,1,2,3
T0
REF
A
B
C
SEINE

Fig. 3

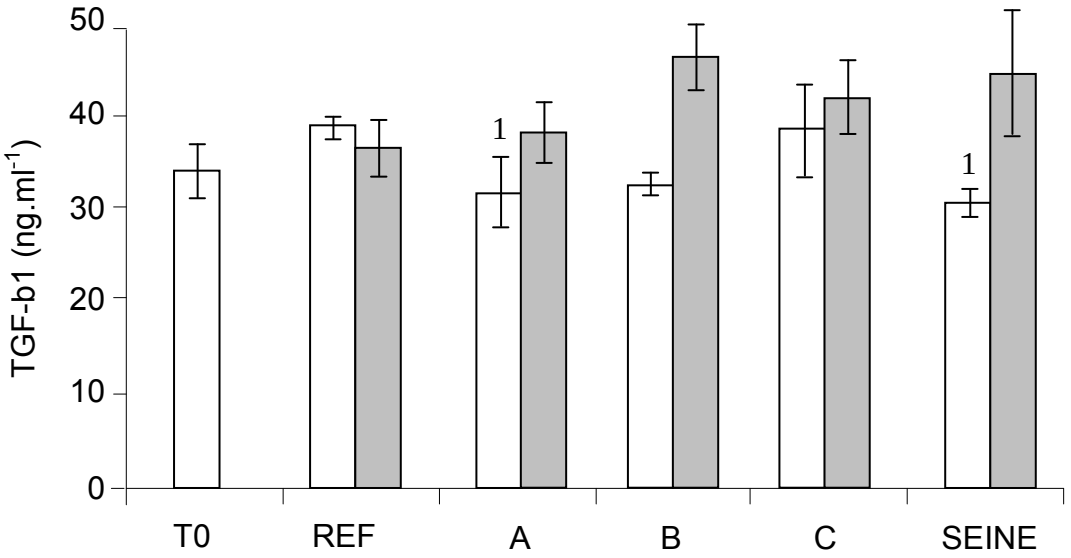
TGF-b1 (ng.ml-1)
50
40
30
20
10
0
1
1
T0
REF
A
B
C
SEINE

Fig.4.

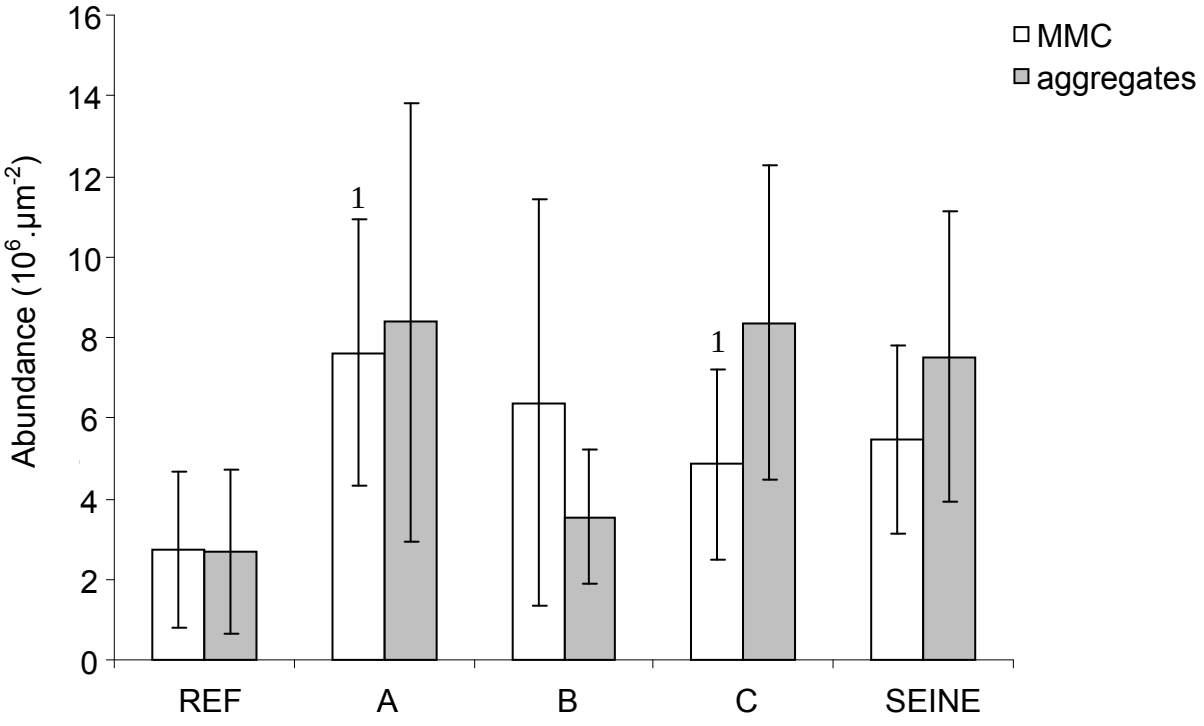
MMC
aggregates
Abundance (10^6.µm^-2)
16
14
12
10
8
6
4
2
0
1
1
REF
A
B
C
SEINE

Fig. 5.

Tables

Table 1

Relationships (correlation coefficients) between molecular, immunological and physiological biomarkers and the levels of metal and PAH concentrations in sediment (n=75 for molecular and physiological biomarkers, n=15 for TGF-b1 and n=40 for MMC and aggregates -Agg). Significant correlation for p* < 0.05, p** < 0.01 and p*** < 0.001.

	----------t_7----------				----------t_{21}----------					
	EROD	GST	CAT	TGF-b1	EROD	GST	CAT	TGF-b1	MMC	Agg
Cd	0.65***	0.01	0.56***	-0.09	0.21	0.40***	0.74***	0.60*	-0.07	0.21
Cr	0.31***	-0.04	0.28*	-0.13	0.36***	-0.05	0.27*	0.55*	0.26	0.20
Cu	0.01	-0.06	-0.01	0.47	0.20	-0.33***	-0.10	0.10	0.27	-0.02
Hg	0.48***	0.01	0.37***	-0.19	0.26*	0.22	-0.55***	0.66**	-0.03	0.11
Mn	0.70***	0.05	0.61***	-0.10	0.11	0.58***	0.85***	0.54*	-0.21	0.16
Ni	0.36***	-0.02	0.32***	-0.01	0.32***	0.01	0.34***	0.52*	0.22	0.17
Pb	0.51***	0.00	0.43***	0.05	0.26*	0.20	0.54***	0.56*	0.06	0.14
V	0.40***	-0.03	0.35***	0.11	0.30**	0.03	0.36***	0.44	0.20	0.18
Zn	0.27*	-0.02	0.22	0.35	0.23	-0.07	0.23	0.32	0.17	0.04
PAHs	0.16	-0.02	0.09	0.15	0.31***	-0.18	0.13	0.45	0.22	0.03

Table 2

Relationships (correlation coefficients) between molecular, immunological and physiological biomarkers measured after 7 (a.) and 21 days (b.) of exposure (n=75 for physiological and molecular biomarkers, n=15 for TGF-b1 and n=40 for MMC). Significant correlation for p* < 0.05, p** < 0.01 and p*** < 0.001.

t_7	EROD	GST	CAT	TGF-b1
GST	-0.1	-		
CAT	0.70***	-0.07	-	
TGF-b1	-0.05	-0.25	-0.21	-
GW	-0.19	-0.02	-0.26*	0.09
GL	-0.16	-0.06	-0.27*	0.04
K	-0.18	-0.02	-0.25*	-0.17
RNA:DNA	-0.18	0.15	-0.14	0.08

a.

t_{21}	EROD	GST	CAT	TGF-b1	MMC	Aggregates
GST	-0.11	-				
CAT	0.01	0.63***	-			
TGF-b1	0.05	0.38	0.45	-	-	-
MMC	0.13	-0.25	-0.19	-	-	-
Aggregates	-0.24	-0.01	0.21	-	-	-
GW	0.01	-0.16	-0.32*	-0.50	-0.18	-0.49*
GL	0.06	-0.17	-0.30*	-0.26	-0.04	-0.48*
RG	-0.14	0.19	0.14	0.09	-0.01	0.01
K	0.14	-0.25	-0.36**	-0.53*	-0.09	-0.31
RNA:DNA	-0.33*	0.05	-0.12	-024	-0.20	-0.18
TAG:ST	-0.10	-0.7	-0.08	-0.38	-0.23	-0.24

b.